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Quantification and histochemical localization of ascorbic acid in 'Delicious,' 'Golden Delicious,' and 'Fuji' apple fruit during on-tree development and cold storage

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ABSTRACT

Apple fruit are subject to multiple stressors during pre- and post-harvest development. Stress-induced reactive oxygen species (ROS) can be detrimental to the fruit, and ascorbic acid (AsA) is involved in many of the antioxidant pathways that detoxify ROS. An inclusive study to characterize AsA dynamics in 'Delicious,' 'Golden Delicious,' and 'Fuji' apples during pre- and post-harvest development was performed. AsA was quantified in fruit harvested prior to, at, and following attainment of physiological maturity. Fruit harvested at physiological maturity was stored in air at 0.5 °C and AsA was monitored at monthly intervals. AsA content in peel exceeded that in cortex for all cultivars at all sampling points. AsA in cortex declined early in fruit development and remained low relative to peel throughout development and storage. During development there was a slight increase in the quantity of AsA at physiological maturity, which correlates with an increase in internal ethylene. 'Delicious' apples harvested at 161 d after full bloom (DAFB) had the highest quantity of AsA, followed by 'Golden Delicious' at 149 DAFB and 'Fuji' at 178 DAFB. AsA localization in fruit sections stained with silver nitrate supported the analytical data obtained via HPLC and revealed AsA localizes to the core line and vascular bundles later in fruit development, and this pattern continues during cold storage.

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1. Introduction

Many metabolic processes, including chloroplastic, mitochondrial and plasma membrane-linked electron transport systems of higher plants, produce reactive oxygen species (ROS) (Apel and Hirt, 2004; Foyer et al., 1994). A plethora of biotic and abiotic environmental factors that induce plant stress can enhance ROS generation (Apel and Hirt, 2004). Systems within a plant to alleviate some of the results of these stresses include the xanthophyll cycle and a number of antioxidant compounds including, but not limited to, α-tocopherol, vitamin E, β-carotene, and glutathione (Apel and Hirt, 2004). The xanthophyll cycle dissipates excess light excitation energy as heat in the antenna complexes, thereby protecting the reaction centers from photooxidation (Foyer et al., 1994). Essential to the function of the xanthophyll cycle is a key antioxidant: L-ascorbic acid (AsA, vitamin C). AsA is one of the most abundant antioxidants in plants and is a cofactor of many plant dioxygenases (Davey et al., 2000).

Fruit, including apple, experience stresses during growth and development in the field as well as at harvest and in the post-harvest environment (processing, storage and transport) (Davey et al., 2000). Antioxidants may play a significant role in maintaining the integrity of apple fruit during development on the tree and throughout storage. Davey et al. (2007) have reported that mean fruit AsA and total AsA content are influenced by harvest dates. Most studies describing AsA dynamics in apples have focused on content at commercial harvest (Lee et al., 2003) or on total antioxidants consisting mostly of phenolics and flavonoids (Eberhardt et al., 2000; Lee et al., 2003; Wolfe et al., 2003). Certain cultivars have been shown to be 'low AsA' and to lose AsA quickly following harvest – i.e. within 10 d (Davey et al., 2007). While AsA dynamics have been well characterized in foliar tissues of apple (Davey et al., 2004; Sircelj et al., 2005) and extensively in other plants (Álvarez-Fernández et al., 2004; Chen and Gallie, 2006; Dolatabadian et al., 2008; Mieda et al., 2004; Sanmartin et al., 2003; Yang et al., 2007), the ascorbic acid–glutathione (AsA–GSH) cycle functions differently in fruit compared to foliar tissue as there is a strong correlation between time of harvest and mean fruit AsA but not time of harvest and GSH (Davey et al., 2007).

Insufficient content of an essential antioxidant may contribute to the development of physiological disorders in apple fruit, partic-

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ularly fruit stored for extended periods (9–12 months). Treatment with exogenous antioxidants reduces the incidence of apple (Jung and Watkins, 2008; Mattheis and Rudell, 2008), pear (Chiriboga et al., 2008), and green pepper fruit (Purvis, 2002) storage disorders. There is a progressive loss of AsA in apple fruit during storage (Davey et al., 2004), and a subsequent increase in the number of physiological storage disorders (Lau, 1998; Watkins et al., 2003, 2004). Factors that contribute to pear fruit core breakdown are also detrimental to fruit AsA quantity (Franck et al., 2003). A positive correlation between chilling injury and AsA quantity in pears and cucumbers has also been demonstrated (Tatsumi et al., 2006). Lower storage temperatures reduce the loss of AsA and the incidence of storage disorders in peas, broccoli and spinach (Favell, 1998).

In 2005, the average U.S. consumer ate an estimated 7.9 kg of fresh-market apples, and 13.2 kg of processed apples, for a total of 20.9 kg of fresh apples and processed apple products (www.usapples.org). AsA in apples as in other plant foods contributes to fruit nutritive value, and low AsA may also impact preference of consumers who are interested in fruit nutritive value. AsA is an essential nutrient as many mammals, including humans and non-human primates, have lost the ability to synthesize AsA due to a highly mutated and non-functional enzyme that controls the last biosynthetic step, L-gulono-1,4-lactone oxidase (Chatterjee, 1973). Also, AsA cannot be stored in the body, so it must be acquired on a regular basis from dietary sources, primarily from plants. In humans, AsA is important for cardiovascular function, immune cell development, connective tissue, and iron utilization. The most vital role within the human body, however, is that of a water-soluble antioxidant (Agius et al., 2003).

Plant tissue staining using $AgNO_3$ has been shown to localize only to the areas where AsA is present, as demonstrated for cucumber root (Liso et al., 2004) and apple fruit (Li et al., 2008). This method may offer an effective means to estimate the quantity of AsA and subsequently storagability and nutritive quality. In the current study, apple fruit slices were stained with $AgNO_3$ to determine if a correlation exists between AsA assessment via visual ($AgNO_3$ staining) and analytical (HPLC) methods.

Crop cultivars with higher antioxidant potential have better stress resistance, nutritional quality, yield, and storage characteristics (Lata et al., 2005). If apple AsA content is related to resistance to physiological disorder development, high AsA content has the potential to both extend storage time and increase fruit nutritional value. Therefore, increasing the understanding of AsA dynamics during pre- and post-harvest development may help to develop production and post-harvest protocols that maintain or enhance AsA content and alleviate ROS toxicity and subsequent loss of marketability and fruit quality. The goal of the current study was to assess the quantity of AsA throughout development and storage of 'Delicious,' 'Golden Delicious,' and 'Fuji' apples.

2. Materials and methods

2.1. Fruit material

'Delicious', 'Golden Delicious', and 'Fuji' apples [*Malus sylvestris* var. *domestica* (Borkh.) Mansf.] were harvested in 2006 in the Washington State University experimental orchard located in Wenatchee, WA. Defect-free 'fruit were harvested weekly beginning 4 weeks prior to and continuing 4 weeks after attainment of physiological maturity. Fruit were harvested at physiological maturity, noted when the internal ethylene concentration began to rise along with an approximate starch index for 'Delicious' of 3, 'Golden Delicious' of 4 and 'Fuji' of 4.5, was stored at 0.5 °C in air for 6 months.

At each harvest and evaluation after storage, 42 fruit were used as follows: 20 for maturity tests, 2 for histochemical staining with silver nitrate, and samples for HPLC analysis were collected from the remaining 20 fruit. Fruit in storage were sampled monthly for 6 months.

2.2. Maturity analysis

Fruit respiration, internal ethylene concentration, flesh firmness, and starch score were evaluated the day of harvest. Stored fruit was allowed to warm to 20 °C prior to analysis. Respiration analysis was conducted using four replicates of five fruit placed into 3.79 L glass jars sealed with teflon lids with two gas ports. Jars were purged with air at 1.7 mL s^{-1} for 1 h, then 1 mL head space gas was collected from the outlet port and analyzed for CO_2 content using a Hewlett Packard 5890 gas chromatograph (Agilent, Palo Alto, CA) equipped with a 0.5 m, 3.2 mm i.d. stainless steel column packed with Porapak Q (Supelco, Bellefonte, PA), a methanizer (John Booker & Co., Austin, TX), and a flame ionization detector. The N_2 carrier, H_2 and air flows were 0.5, 0.5, and 5 mL s^{-1}, respectively. Oven and injector temperatures were 35 and 300 °C, respectively. The methanizer temperature was 290 °C controlled by an Instrumentation Temperature Controller (Valco Instruments, Inc., Houston, TX) with an H_2 flow of 0.5 mL s^{-1}.

Internal ethylene concentrations (IECs) were measured by withdrawing 1 mL of gas from the core of each (n = 20) fruit (Williams and Patterson, 1962). Gas samples were injected into a Hewlett Packard 5880 gas chromatograph (Agilent, Palo Alto, CA) equipped with a 0.5 m, 3.2 mm i.d. glass column packed with Porapak Q (Supelco, Bellefonte, PA) and a flame ionization detector. The N_2 carrier, H_2, and air flows were 0.5, 0.2, and 1.7 mL s^{-1}, respectively. Oven, injector and detector temperatures were 60, 100, and 200 °C, respectively.

Flesh firmness was assessed with a penetrometer (n = 20) (Mohr Digi-Test, Mohr & Associates, Richland, WA), equipped with a cylindrical plunger 11 mm in diameter. The measurement was obtained on the fruit equator following removal of peel.

The extent of starch hydrolysis (n = 20) was rated visually using a 1–6 scale (1 = full starch, 6 = no starch) after staining an equatorial section of each apple with a 5 mg L^{-1} I–KI solution.

2.3. Ascorbic acid extraction

The procedure of Davey et al. (2003) was used with modification. Peel tissue was removed using an A505 Peel Away Apple Peeler (Back to Basics, Bluffdale, UT). The core was separated from the cortex and discarded. Three replicates, each between 30 and 50 g, fresh weight, of peel and cortex tissues were frozen using liquid nitrogen and stored at −80 °C. Prior to analysis, tissues were freeze dried, then ground to a fine powder using an IKA A11 Basic S1 grinder (IKA Works, Inc., Wilmington, NC). Approximately 50 mg of the powder was weighed and placed in a 2 mL microcentrifuge tube to which 1 mL chilled extraction buffer consisting of 750 mM meta-phosphoric acid (MPA), 2 mM EDTA, and 10 g L^{-1} insoluble PVPP was added. Tubes were centrifuged at 14,000 × g for 10 min in an Eppendorf 5415D centrifuge (Eppendorf North America, Inc., Westbury, NY). The supernatant was transferred to a 2 mL volumetric flask, and the pellet was resuspended in an additional 1 mL of extraction buffer, vortexed, centrifuged as previously described, and then the supernatants were combined. The final volume of the extract was brought to 2 mL using dH_2O. Following additional centrifugation at 14,000 × g to remove all remaining tissue from solution, 125 μL of supernatant was placed in a 13 mm × 100 mm test tube and 125 μL of chilled (4 °C) reduction buffer consisting of 200 mM DTT and 400 mM Trizma base was added. Tubes were incubated at 25 °C for 4 h and the reaction was stopped with the addition of 125 μL of 8.5% phosphoric acid. Aliquots of this solution were filtered through a disposable 0.45-μm PTFE membrane (Whatman, Florham Park, NJ) and then analyzed by HPLC.

A recovery experiment was performed to test the applicability of freeze drying the tissue prior to AsA extraction. Cortex and peel of five apples were frozen in LN_2 then placed in −80 °C for 24 h. Half of the tissues were then freeze dried overnight, then all tissues were ground in LN_2 and extracted as described.

2.4. Ascorbic acid analyses

Samples (30 μL) were analyzed using a reverse-phase HPLC system (Series 1100 Hewlett Packard, Santa Clara, CA) consisting of an injector, a Hypersil ODS guard column (5 μm, 4.0 mm × 4 mm; Agilent Technologies, Santa Clara, CA), a Hypersil ODS column (5 μm, 4.0 mm × 125 mm; Agilent Technologies, Santa Clara, CA) and a photodiode array (PDA) detector (model 996; Waters, Milford, MA) that employed a Series 1100 Hewlett Packard autosampler. The system was controlled and the data was analyzed using Millennium[32] software (Waters, Milford, MA). Ascorbic acid was eluted at a flow rate of 16.7 $\mu L\,s^{-1}$ and a column temperature of 30 °C using a binary gradient modified from Davey et al. (2003) that consisted of 100% solvent A (0.25% MeOH, 0.1 mM EDTA, 400 $\mu L\,L^{-1}$ *o*-phosphoric acid) for the first 2 min, then solvent B (0.25% MeOH, 0.1 mM EDTA, 400 $\mu L\,L^{-1}$ *o*-phosphoric acid, 30% acetonitrile) increased linearly and reaching 100% at 3 min then held for 3 min. The AsA peak was identified by retention time and spectral comparison to an authentic standard prepared in 375 mM MPA, 1 mM EDTA and 2.5 mM DTT. The standard solution was stored at −20 °C and used to calibrate the detector daily. AsA was quantified at 243 nm.

2.5. Histochemical localization of AsA using $AgNO_3$ staining

AsA localization was carried out as described by Chinoy (1984) and Liso et al. (2004). Apples were allowed to warm to room temperature, then an equatorial slice was completely immersed in staining solution. Tissue was incubated in the dark at 4 °C with acidified $AgNO_3$ solution consisting of 5% (w/v) $AgNO_3$, 66% (v/v) absolute ethanol and 5% (v/v) glacial acetic acid for 5 d. Black deposits of metallic silver form where AsA is located. The reaction was stopped with an ammonia wash (5 mL ammonia in 95 mL 70% ethanol). Digital photographs were taken to record the staining pattern.

2.6. Statistical analysis

All AsA measurements were made in triplicate and the means regressed against DAFB or months in storage, as appropriate, to evaluate significance ($p < 0.05$) of linear and/or polynomial fits using the general linear model (Proc GLM, SAS Version 9.1, SAS Institute, Cary, NC).

3. Results

Based on internal ethylene concentration (IEC), firmness, and starch index, physiological maturity had been attained by 149, 161, and 178 DAFB for 'Golden Delicious,' 'Delicious,' and 'Fuji,' respectively (Tables 1–3). 'Delicious' IEC began to increase at 153 DAFB and exceeded 1 $\mu L\,L^{-1}$ at 168 DAFB and thereafter. Starch scores exceeded 2 at and after 161 DAFB. 'Golden Delicious' IEC increased at 149 DAFB but did not exceed 1 $\mu L\,L^{-1}$ until 171 DAFB, starch score increased from 1.8 to 3.9 between 143 and 149 DAFB. 'Fuji' IEC was greater than 0.5 1 $\mu L\,L^{-1}$ and starch score averaged 4.3 at 178 DAFB. Firmness of all three cultivars decreased during the harvest period.

Table 1
Progression of 'Delicious' fruit maturity in 2006. Date of physiological maturity is indicated (*). Fruit were harvested in a research orchard located in Wenatchee, WA. Analysis was conducted the day of harvest. Values are means followed by standard deviations ($n = 20$).

Harvest date	9/11/2006	9/18/2006	9/24/2006	10/1/2006	10/9/2006*	10/16/2006	10/23/2006	10/29/2006	11/5/2006
DAFB	133	140	146	153	161	168	175	178	188
Respiration rate, CO_2 production, $mol\,kg^{-1}\,s^{-1}$	[a]	0.062 ± 0.004	0.0039 ± 0.003	0.074 ± 0.004	0.0050 ± 0.002	0.033 ± 0.001	0.031 ± 0.003	0.095 ± 0.011	0.16 ± 0.008
Internal ethylene, $\mu L\,L^{-1}$	0.000 ± 0.00	0.004 ± 0.01	0.001 ± 0.00	0.039 ± 0.08	0.055 ± 0.21	6.13 ± 10.83	2.80 ± 5.56	29.5 ± 31.25	8.23 ± 9.83
Weight (g)	162.3 ± 23.1	160.4 ± 30.9	163.7 ± 19.9	180.6 ± 26.6	201.2 ± 20.0	223.9 ± 28.4	202.2 ± 22.0	182.8 ± 23.9	250.5 ± 39.5
Firmness (N)	80.1 ± 4.5	80.0 ± 4.3	76.5 ± 0.8	75.2 ± 4.2	70.6 ± 1.3	68.0 ± 1.2	63.1 ± 2.3	58.2 ± 8.7	51.0 ± 1.7
Starch (1–6 scale)	1.1 ± 0.1	1.4 ± 0.3	1.2 ± 0.0	1.9 ± 0.2	2.8 ± 1.2	2.6 ± 0.8	3.5 ± 1.2	5.6 ± 0.3	4.8 ± 1.0

[a] Not measured.

Table 2
Progression of 'Golden Delicious' fruit maturity in 2006. Date of optimum harvest is indicated (*). Fruit were harvested in a research orchard located in Wenatchee, WA. Analysis was conducted on day of harvest after fruit warmed to room temperature. Values are means followed by standard deviations ($n = 20$).

Harvest date	9/6/2006	9/11/2006	9/18/2006	9/24/2006*	10/1/2006	10/9/2006	10/16/2006	10/23/2006
DAFB	131	136	143	149	156	164	171	178
Respiration rate, CO_2 production, mol kg^{-1} s^{-1}	0.069 ± 0.003	0.0028 ± 0.003	0.053 ± 0.006	0.069 ± 0.003	0.11 ± 0.006	0.039 ± 0.006	0.078 ± 0.011	0.061 ± 0.008
Internal ethylene μL L^{-1}	0.004 ± 0.01	0.001 ± 0.01	0.001 ± 0.01	0.04 ± 0.13	0.05 ± 0.10	0.15 ± 0.32	7.8 ± 7.98	4.3 ± 7.65
Weight (g)	168.8 ± 18.8	177.6 ± 27.3	164.4 ± 21.6	172.7 ± 28.8	213.4 ± 22.6	214.6 ± 31.8	220.4 ± 29.5	218.3 ± 29.1
Firmness (N)	83.1 ± 5.1	80.7 ± 6.1	74.5 ± 4.7	71.5 ± 4.8	73.9 ± 6.0	72.2 ± 4.3	65.0 ± 6.0	61.5 ± 7.6
Starch (1–6 scale)	1.0 ± 0.1	1.2 ± 0.2	1.8 ± 0.4	3.9 ± 0.9	4.1 ± 0.6	5.6 ± 0.4	5.4 ± 0.5	5.8 ± 0.3

Table 3
Progression of 'Fuji' fruit maturity in 2006. Date of optimum harvest is indicated (*). Fruit were harvested in a research orchard located in Wenatchee, WA. Analysis was conducted on day of harvest after fruit warmed to room temperature. Values are means followed by standard deviations ($n = 20$).

Harvest date	10/1/2006	10/9/2006	10/16/2006	10/23/2006	10/29/2006*	11/5/2006	11/12/2006	11/19/2006	11/26/2006
DAFB	150	158	165	172	178	185	192	199	206
Respiration rate, CO_2 production, mol kg^{-1} s^{-1}	90.0 ± 7.2	0.017 ± 0.003	0.042 ± 0.003	0.028 ± 0.003	0.094 ± 0.003	0.14 ± 0.006	0.092 ± 0.006	0.092 ± 0.006	0.078 ± 0.02
Internal ethylene, μL L^{-1}	0.000 ± 0.00	0.03 ± 0.07	0.48 ± 0.72	0.056 ± 0.13	0.57 ± 0.78	0.42 ± 0.76	2.8 ± 3.08	8.1 ± 6.84	3.5 ± 11.60
Weight (g)	192.7 ± 28.9	228.7 ± 38.3	250.1 ± 37.6	231.3 ± 34.4	226.5 ± 29.7	248.2 ± 33.8	238.9 ± 30.9	237.6 ± 35.5	218.9 ± 37.0
Firmness (N)	76.5 ± 5.7	73.8 ± 6.2	68.0 ± 6.2	66.3 ± 4.3	69.0 ± 8.2	58.0 ± 4.4	59.4 ± 5.0	59.0 ± 5.6	61.8 ± 6.3
Starch (1–6 scale)	1.6 ± 0.3	2.6 ± 0.5	2.5 ± 0.8	3.6 ± 0.8	4.3 ± 1.0	5.8 ± 0.1	5.9 ± 0.2	6.0 ± 0.1	6.0 ± 0.0

Table 4
Progression of 'Delicious,' 'Golden Delicious,' and 'Fuji' fruit ripening during air storage for 6 months at 0.5 °C. Fruit was harvested 161, 149, and 178 d after full bloom, respectively, in a research orchard in Wenatchee, WA. Upon removal from storage, fruit was allowed to warm to room temperature before analysis. Values are means followed by standard deviations (n = 20).

	Months in storage					
	1	2	3	4	5	6
'Delicious'						
Respiration rate, CO_2 production, mol kg^{-1} s^{-1}	0.11 ± 0.003	0.097 ± 0.003	0.13 ± 0.003	0.10 ± 0.01	0.16 ± 0.01	0.033 ± 0.006
Internal ethylene, μL L^{-1}	1.23 ± 34.3	62.1 ± 75.7	64 ± 108.3	54 ± 62.9	32 ± 77.0	0.59 ± 2.4
Firmness (N)	69.2 ± 7.1	60.5 ± 5.2	50.6 ± 4.5	47.5 ± 5.3	46.1 ± 4.3	43.4 ± 3.6
Starch (1–6 scale)	3.6 ± 1.1	5.6 ± 0.3	5.8 ± 0.2	6.0 ± 0.1	6.0 ± 0.0	6.0 ± 0.0
'Golden Delicious'						
Respiration rate, CO_2 production, mol kg^{-1} s^{-1}	0.17 ± 0.01	0.086 ± 0.006	0.14 ± 0.003	0.072 ± 0.01	0.22 ± 0.008	0.061 ± 0.003
Internal ethylene, μL L^{-1}	90.0 ± 73.1	123.0 ± 144.2	26.5 ± 30.5	67.4 ± 83.4	183.6 ± 247.3	36.0 ± 59.2
Firmness (N)	60.2 ± 3.7	53.3 ± 5.8	49.8 ± 6.4	49.2 ± 7.6	42.8 ± 5.6	45.9 ± 6.0
Starch (1–6 scale)	5.6 ± 0.6	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.1	6.0 ± 0.0	6.0 ± 0.0
'Fuji'						
Respiration rate, CO_2 production, mol kg^{-1} s^{-1}	0.067 ± 0.008	0.11 ± 0.003	0.11 ± 0.01	0.21 ± 0.006	0.22 ± 0.008	0.24 ± 0.01
Internal ethylene, μL L^{-1}	21.7 ± 37.3	74.8 ± 59.3	17.9 ± 24.1	11.8 ± 24.3	24.1 ± 51.5	5.3 ± 16.9
Firmness (N)	68.4 ± 3.9	63.6 ± 4.6	63.5 ± 4.9	63.6 ± 7.6	65.1 ± 5.4	58.9 ± 7.7
Starch (1–6 scale)	5.8 ± 0.3	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0

'Delicious' respiration rate was fairly constant for the first 5 months of storage followed by a decrease at 6 months (Table 4). 'Golden Delicious' respiration rate after storage was variable with a peak after 5 months, and 'Fuji' respiration increased after month 4. 'Delicious' ethylene evolution increased from month 1 to 2, then decreased from months 3 to 6. All starch was converted by month 4. 'Golden Delicious' ethylene production was variable with the highest value after 5 months, all starch was converted after 2 months. Highest IEC and complete conversion of starch for 'Fuji' was observed at 2 months of storage. 'Fuji' IEC values were generally lower compared to 'Delicious' and 'Golden Delicious'. Firmness for both 'Delicious' and 'Golden Delicious' declined sooner as storage duration increased compared to 'Fuji'.

Freeze drying did not alter AsA recovery (data not shown). Ascorbic acid content in cortex and peel decreased during development relative to values during the first several weeks after bloom in all cultivars (Fig. 1). Cortex AsA content was highest at the first harvest date for all cultivars and decreased by 54, 48, and 62% for 'Delicious,' 'Golden Delicious,' and 'Fuji', respectively, between 20 and 30 DAFB. The relationship between DAFB and cortex AsA had a significant relationship for all cultivars. Peel tissue AsA content for all cultivars had a general trend of decreasing from the initial value then increasing after ~ 130 DAFB. The later peak in peel AsA content occurred during the period where physiological maturity was attained. Peel AsA content and harvest date in 'Delicious' had a cubic relationship, whereas relationships for 'Golden Delicious' and 'Fuji' were quartic.

Cortex AsA and storage duration had cubic and quartic relationships for 'Delicious' and 'Golden Delicious', respectively. During months 3–5 in storage, 'Fuji' had a significantly higher cortex AsA than 'Delicious' and 'Golden Delicious' (t-test $p \leq 0.05$) (Fig. 2). The relationship for 'Fuji' between AsA content and storage duration was quadratic.

Peel AsA contents during storage were also close to that at harvest for all cultivars (Fig. 3). During storage, peel AsA content was approximately 2–5 'Delicious' and twofold 'Golden Delicious' the amount detected in cortex, and both cultivars had a quadratic relationship between peel AsA and storage duration. 'Fuji' peel had approximately four times the amount of AsA compared to cortex AsA content, and a quartic relationship between peel AsA and storage duration.

Histochemical staining patterns for AsA were similar to the analytical results. Staining appears as very discrete dark spots or patches (Fig. 4) or well-defined lines (Fig. 5) in the cortex. The darkened, non-discrete areas are the result of tissue break down during the staining incubation period and not staining per se. Incubations for 5 d were optimum, as shorter incubations provided little staining, and there was a noticeable amount of tissue breakdown with

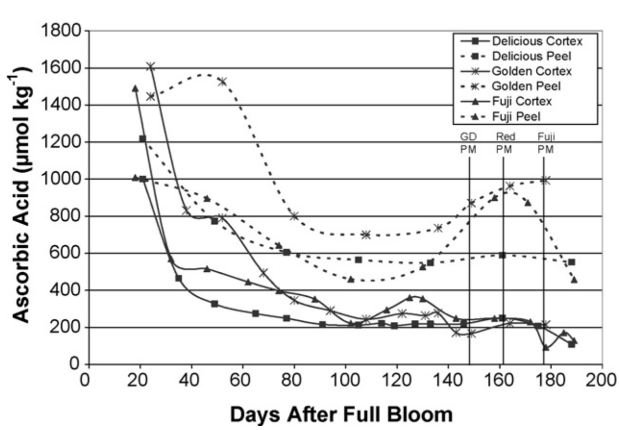

Fig. 1. Ascorbic acid content in 'Delicious,' 'Golden Delicious,' and 'Fuji' apples at harvest. PM = physiological maturity. Values are means (n = 20) on a fresh weight basis.

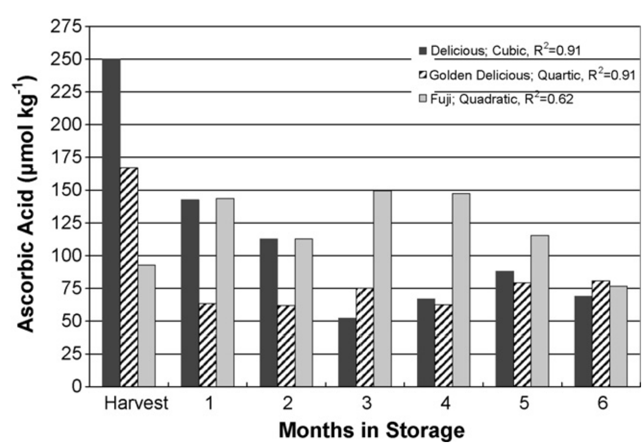

Fig. 2. Ascorbic acid content in 'Delicious,' 'Golden Delicious,' and 'Fuji' cortex tissues following air storage at 0.5 °C. Fruit were harvested at physiological maturity. Highest coefficient of determination (R^2) indicated. Values are means (n = 20) on a fresh weight basis.

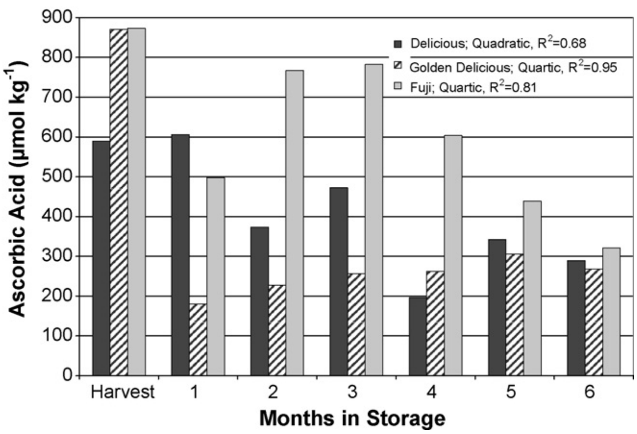

Fig. 3. Ascorbic acid content in 'Delicious,' 'Golden Delicious,' and 'Fuji' peel tissues following air storage at 0.5 °C. Fruit were harvested at physiological maturity. Highest coefficient of determination (R^2) indicated. Values are means (n = 20) on a fresh weight basis.

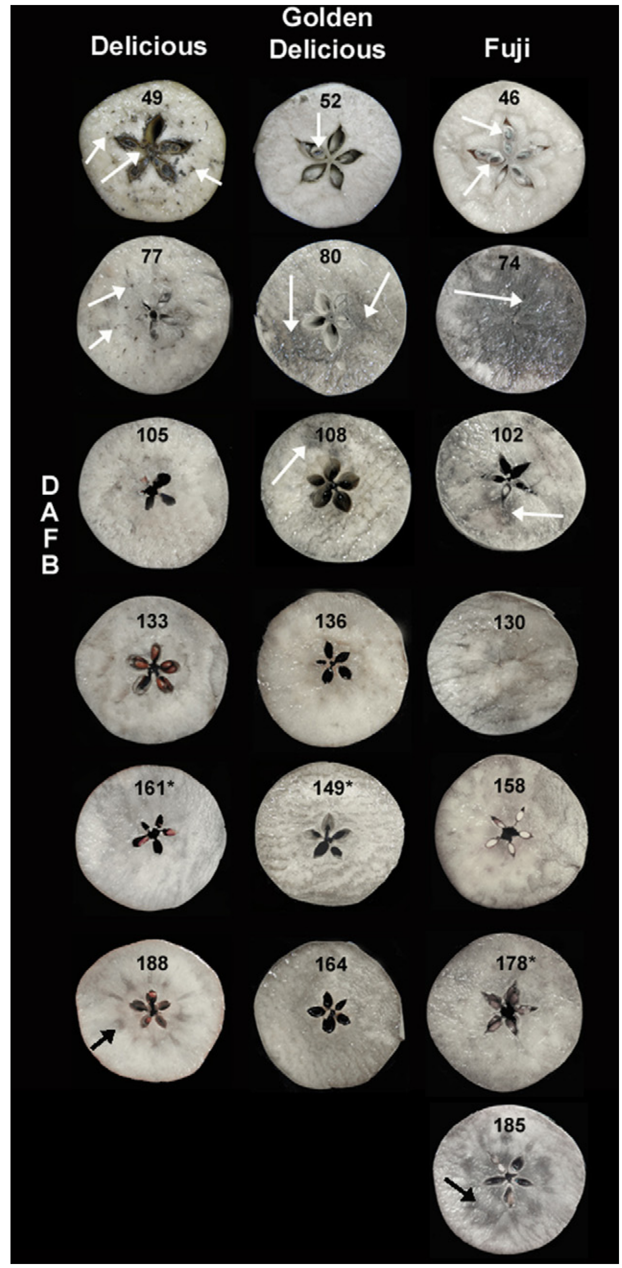

Fig. 4. Histochemical localization by $AgNO_3$ of ascorbic acid in apple tissue at harvest. 'Delicious,' 'Golden Delicious,' and 'Fuji' apples were harvested at weekly intervals. Transverse sections through the equatorial portion of apples were incubated with an $AgNO_3$ solution (5% (w/v) $AgNO_3$, 66% (v/v) absolute ethanol and 5% (v/v) glacial acetic acid) for 5 d at 4 °C. Dark areas, as shown by white arrows, indicate presence of ascorbic acid. Days after full bloom (DAFB) at harvest are indicated on the top of each slice in black, with optimal harvest indicated by asterisk. Black arrows indicate watercore.

longer incubations. For all cultivars, more staining occurred in fruit harvested during the early stages of development, specifically the first two harvest dates, compared to fruit harvested later. In early developmental stages, stained AsA in 'Delicious' cortex appears as discrete dark spots. Discrete spots were visible at 77 DAFB although fewer were observed. At 105 and 133 DAFB, the spots were located around the core. No staining was apparent in samples from the later harvests. At 188 DAFB, the discoloration around the core was due to watercore and not stained tissue.

A large portion of the staining in 'Golden Delicious' harvested at 52 DAFB was present in the seed cavity. At 80 DAFB there were large patches of AsA staining, only a small portion at 108 DAFB, and virtually none at the later sample dates.

The 'Fuji' staining pattern was similar to that of 'Golden Delicious.' There were large AsA patches at 74 DAFB that decreased in intensity with increased DAFB until no staining was visible. 'Fuji' fruit harvested 185 DAFB had watercore, which again should not be confused for AsA staining.

Much of the AsA localized to the core line and not the cortex after storage (Fig. 5). The overall decrease in staining is consistent with the lower AsA quantity analytically determined in stored fruit compared to fruit at harvest. The changes in cortex AsA quantity during storage are small compared to those occurring during development, and the low amount present in stored fruit made visualization through staining difficult. However, for cortex tissue, the staining pattern follows that of the HPLC results for each cultivar. 'Delicious' had a higher quantity of AsA at the beginning of storage, with a slight decrease at 3 months, followed by an increase. The last two harvest dates had a considerable amount of watercore. AsA staining in 'Golden Delicious' and 'Fuji' remain fairly constant throughout storage.

4. Discussion

This inclusive survey of apple AsA in cortex and peel tissue during pre-harvest development as well as during storage confirmed the amount of AsA is strongly correlated with harvest date, as previously shown (Davey and Keulemans, 2004), a trend revealed to be consistent over several years (Davey et al., 2007). Planchon et al. (2004) previously demonstrated that traditional Belgian cultivars have three to seven times more AsA than the current commercial cultivars 'Gala' and 'Elstar,' respectively. High AsA content is a useful trait in apples because antioxidants are critical for plant responses to oxidative stress while also contributing to fruit nutritive value.

Only one growing season was analyzed in the current study because correlations between AsA and harvest date are primarily determined by the genetic background of the cultivar (Davey et al., 2007). In the analytical analyses, all forms of AsA were reduced, therefore the values reported are total AsA within the sample analyzed. Previous studies have quantified total (oxidized and reduced) AsA (Lata, 2007), while others have quantified dehydroascorbic acid (DHA) and AsA. Li et al. (2008) demonstrated the ratio of AsA to DHA remains consistent between young and mature fruit although quantities of both are higher in young fruit. It has also been shown at harvest there is a higher amount of AsA than DHA, and that the

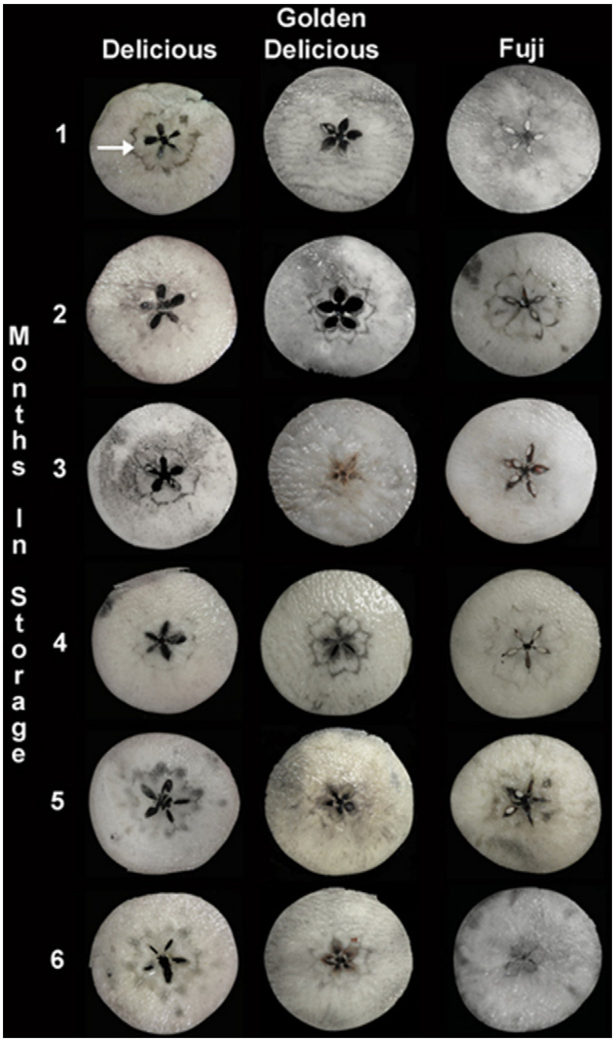

Fig. 5. Histochemical localization by $AgNO_3$ of ascorbic acid in apple tissue after storage. 'Delicious,' 'Golden Delicious,' and 'Fuji' apples were harvested at physiological maturity, stored at 0.5 °C in air and removed monthly for 6 months. Transverse sections through the equatorial portion of apples were incubated with an $AgNO_3$ solution (5% (w/v) $AgNO_3$, 66% (v/v) absolute ethanol and 5% (v/v) glacial acetic acid) for 5 d at 4 °C and documented via digital photography. Dark areas indicate presence of ascorbic acid.

AsA to DHA ratio decreases from the peel to the core (Davey et al., 2004).

The larger quantity of AsA in peel as compared to cortex for all cultivars in this study is consistent with a protective role of the peel to respond to both abiotic and biotic stresses such as ultraviolet radiation, wind, pathogens, and insects. The samples in this study were a combination of sun exposed and shade peel. Ma and Cheng (2004) have shown that in response to exposure to the sun, both the xanthophyll and ascorbate–glutathione cycle are upregulated. In addition, it has been shown that phenolics, which exhibit antioxidant and antiproliferative activities, have a larger role in apple peel than in apple flesh (Eberhardt et al., 2000; Wolfe et al., 2003). Apple peel contains 1.5–9.2 times greater total antioxidant capacity as compared to flesh (Drogoudi et al., 2008). Peel of 'Gala' apples was previously shown to have the highest quantity of AsA compared to other fruit tissues (Li et al., 2008).

The silver staining pattern visually supports the quantitative trends established with HPLC analysis and provides insight as to intra-fruit AsA localization. Unlike apple foliar tissue, cortex and peel tissues are incapable of *de novo* AsA synthesis (Davey et al., 2004), therefore all fruit AsA is transported through the vasculature, specifically the phloem, as demonstrated for *Arabidopsis thaliana* and *Medicago sativa* (Franceschi and Tarlyn, 2002). The staining pattern indicating AsA increasingly localized to the core line and vascular bundles during development of 'Delicious' and 'Golden Delicious' and the relatively low AsA content in all three cultivars during storage is consistent with a foliar origin for apple fruit AsA. 'Fuji' had more staining overall with a notable increase at 74 DAFB detected by both HPLC and silver staining. Throughout storage for all cultivars there was an overall low quantity of AsA, as seen by the HPLC, which was supported by the silver staining. In later developmental stages and during storage the majority of the staining was localized to the core line and vascular bundles, which is consistent with results for 'Gala' (Li et al., 2008). The low values during storage indicate certain apples may be more susceptible to storage disorders than others, depending on the quantity upon entering storage.

Davey et al. (2004, 2007), has shown a strong negative correlation between date of harvest and mean fruit AsA, a correlation not immediately discernable in the current study. This correlation is expected since the fruit are a sink, with no capacity for synthesis. The values for leaves near the fruit on the tree have very little change in their AsA quantity during development of the fruit, however (Davey et al., 2004). The order of AsA quantity at harvest was highest for 'Delicious,' followed by 'Golden Delicious,' and lowest for 'Fuji' with physiological maturities occurring 161, 149 and 178 DAFB, respectively. Regression analysis seems to indicate there are different patterns over developments, which are dependent on cultivar. Individually, each cultivar followed the trend of decreasing AsA as DAFB increased. The decrease may be attributed to sink–source relations within the tree. AsA enters the fruit through the vasculature, and diffuses from that point. As time progresses, it is plausible that it utilizes less AsA, therefore imports less, and the decrease becomes apparent. These cultivars were not evaluated by Davey et al. (2004, 2007), and have different genetic origins, so slight differences, such as 'Golden Delicious' having a slightly higher quantity of AsA than 'Delicious,' are expected. In addition, Selvarajah et al. (2001) discovered that 1-MCP reduced the rate of AsA decline in fruits and Vilaplana et al. (2006) 1-MCP gave apples greater enzymatic antioxidant potential, suggesting that ethylene may be involved in the metabolism of AsA. Indeed, the peak of total AsA (cortex and peel) at physiological maturity only slightly precedes the peak in ethylene during on-tree ripening.

The quantity of water-soluble antioxidants in apple peel has previously been shown to decrease during storage while content of lipid soluble antioxidants increases (Barden and Bramlage, 1994). In addition, Davey et al. (2007) reported that early ripening cultivars lose AsA more rapidly during storage compared to late ripening cultivars, a pattern confirmed in the current work. The decline in AsA content may be due, in part, to the progression of fruit ripening. The gradual loss of AsA throughout storage observed with the three cultivars in the current study is consistent with previous reports. Harvest removes fruit from the AsA source (leaves), and AsA content gradually decreases during storage. $AgNO_3$ staining indicated depletion of AsA in the vascular bundles lags other tissues possibly indicating a slower utilization rate and/or a higher content at harvest in this tissue. However, there appears to be no relationship between harvest date and the capacity of 'Delicious', 'Golden Delicious', and 'Fuji' apples to maintain AsA during storage (Davey et al., 2007).

5. Conclusions

The three cultivars examined differed in AsA content, and the quantity of AsA during development is higher compared to content during storage. Peel had an overall higher quantity of AsA versus cortex, but there was a decline in quantity for both tissues. Silver staining proved useful in visualizing the location of AsA in the fruit interior tissues. This method could possibly be modified for rapid use in quick AsA quantification.

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